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Vasoreactivity in an adult rat model of marginal copper deficiency

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Abstract

Short-term severe copper deficiency has been shown to significantly reduce acetylcholine (Ach)-induced vascular smooth muscle relaxation. The current study was designed to examine the long-term relationship of marginal dietary Cu to vasoreactivity. Male adult rats were fed a purified diet with adequate (6.0 μg) or marginal (3.0 or 1.5 μg) Cu/g diet for 6 months. Luminal diameter changes were measured in isolated resistance arterioles. Liver and kidney Cu concentrations were used as indices of Cu status. The results showed a significant decrease in kidney Cu in the 1.5-μg group compared with the adequate controls but no effect on liver Cu. There was a significant correlation between dilation to 10^{-6} mol/L Ach and kidney Cu but no relationship between Cu status and 10^{-5} mol/L norepinephrine-induced constriction or flow-induced dilation. There was also no dietary effect on baseline vessel tone or dilator capacity. The data are the first to establish a linear relationship between Ach-induced vasodilation and systemic Cu status in a mature long-term model of marginal Cu deficiency.
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1. Introduction

Severe dietary copper has been shown to affect vascular smooth muscle reactivity to both constrictor and dilator agonists. Aortic contraction to norepinephrine (NE) is augmented in

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severely Cu-deficient rats [1] as is contraction of smaller resistance vessels to angiotensin II in isolated lungs from Cu-deficient rats [2]. This effect is apparently selective in that an altered constrictor response to NE was absent when skeletal muscle microcirculation of Cu-deficient rats was observed in vivo [3].

In addition to constrictor effects, previous studies have demonstrated that severe Cu deficiency attenuates dilation to acetylcholine (Ach) and other nitric oxide (NO)–mediated dilators in both large conduit arteries [4,5] and in small resistance arterioles of the microcirculation [3,6-8]. Similar impaired dilation to Ach has been reported in isolated vascular rings when Cu has been chelated [9].

Although dilation to Ach is affected during Cu deficiency, we have shown that dilation to the second messenger analogs dibutyryl cGMP and dibutyryl cAMP or to the phosphodiesterase inhibitor papaverine is not inhibited in the Cu-deficient rat [3]. These results suggest that the vascular smooth muscle relaxation mechanisms and dilator capacity are not altered by Cu deficiency. Thus, the impaired dilation to Ach during Cu deficiency is a result of Cu dependency within the Ach-NO signal trans-duction pathway and not because of a diminished capacity of the vascular smooth muscle to relax [4-6,9].

The protocol used to study vasoreactivity and Cu nutrition in the above dietary studies used diets very low in Cu content (<1 mg/kg, as compared with the requirement of 5 mg/kg for rodents [10]) fed to young, rapidly growing animals. Whereas these studies of short-term, severe Cu deficiency in weanling animals support the essentiality of Cu for Ach-stimulated vasodilation, the current study was designed to determine whether such function is compromised in marginally Cu-deficient adult animals. This represents a logical extension of prior work in that marginal intakes more closely represent diets that human beings might consume [11,12] and, if dietary Cu deficiency is to be regarded as a potential contributor to cardiovascular disease, such disease is more likely to manifest itself in adults.

Marginal Cu deficiency is difficult to discern, even when initiated in young animals that are most susceptible to Cu depletion [13-15] Although consequences have been attributed to the feeding of marginally low Cu diets [14,15], the absence of direct and sensitive indices makes it difficult to attribute cause and effect. The difficulty in finding sensitive indices of Cu status was illustrated in a study using young rats, which showed that neither liver Cu nor serum ceruloplasmin, a Cu-dependent enzyme, 2 commonly used status indices for severe deficiency, were effective in delineating Cu status over marginal ranges of intake [13]. Furthermore, we have found that adult rats are difficult to make Cu deficient, even with a severely deficient diet, apparently because their stores have been established. From these considerations, it is logical to anticipate that any potential effects of marginal deficiency are likely to be subtle and slow to develop, that is, they will look similar to cardiovascular disease in human beings. Therefore, such study will logically require sensitive indices, experiments of long duration, and relatively large numbers of subjects. These requirements were satisfied in the present study by using a sensitive index, kidney Cu, derived from the earlier study of young animals [13], by studying marginal deficiency over a period of 6 months and by using mathematical regression techniques that allow use of animals from all dietary groups to calculate a relationship.

2. Methods and materials

2.1. Animals and diet

This project was approved by the University of Louisville Animal Care and Use Committee. Male adult Sprague-Dawley rats (8 weeks old, 250 g) were purchased from Charles River Breeding Laboratories (Wilmington, Mass). On arrival, rats were housed individually in stainless steel cages in a temperature- and humidity-controlled room with a 12-h light-dark cycle. The rats were given free access to distilled water and to 1 of 3 purified diets for 6 months. The basal diet was a casein-sucrose-cornstarch-based diet (no. TD 84469, Teklad Test Diets, Madison, Wis) containing all known essential vitamins and minerals except for Cu and iron. The Cu-adequate diet ($CuA_{6.0}$) consisted of the basal diet (940 g/kg of total diet) with safflower oil (50 g/kg) and a copper-iron mineral mix that provided 0.22 g of ferric citrate (16% Fe) and 24 mg of $CuSO_4 \cdot H_2O$ per kilogram of diet, thus providing about 6.0 mg copper/kg diet. The copper-marginal diets were the same except for partial replacement of copper with cornstarch in the mineral mix to provide 3.0 ($CuM_{3.0}$) and 1.5 ($CuM_{1.5}$) mg Cu/kg diet. Diet analysis by atomic absorption spectrophotometry indicated that the Cu-adequate and -marginal diets contained 5.88, 2.94, and 1.62 mg Cu/kg diet, respectively. Parallel assays of National Institute of Standards and Technology (Gaithersburg, Md) reference samples (citrus leaves, no. 1572) yielded values within the specified range, which validated our copper assays.

2.2. Organ Cu determination

The median lobe of the liver and the right kidney were removed, weighed, and frozen at $-20°C$ for subsequent Cu analysis. Tissues were lyophilized and digested in nitric acid and hydrogen peroxide [16]. Organ Cu concentrations of individual rats were assessed by using inductively coupled argon plasma emission spectrometry (Jarrell-Ash, model 1140, Waltham, Mass). Parallel assays of reference samples (no. 1477a, bovine liver) from the National Institute of Standards and Technology yielded mineral contents within the specified range.

2.3. Vasoreactivity

Experiments were conducted using isolated, perfused resistance arterioles from the rat cremaster muscle microcirculation. Eight or nine rats from each of the dietary groups were anesthetized with sodium pentobarbital (50 mg/kg) via intraperitoneal injection. The cremaster muscle was excised and placed in a refrigerated well (0°C-4 °C) containing a solution of (in mmol/L) 145 NaCl, 5.0 KCl, 2.0 CaCl, 1.0 $MgSO_4$ 1.0 NaH_2PO_4, 5.0 dextrose, 2.0 pyruvate, 0.02 disodium ethylenediamine tetraacetate (Na_2EDTA), and 3.0 3-(*N*-morpholino)propanesulfonic acid–physiological saline solution (MOPS-PSS; pH 7.4 $\pm$ 0.3) as well as 1% albumin [17]. A segment of the A1 arteriole was excised, cannulated with glass micropipettes, and secured to the pipettes with 12-0 suture. MOPS-PSS without albumin was used to bath the vessel. The arterioles were pressurized to 90 cm H_2O (approximate in vivo pressure [18]). The cannulation pipettes had equivalent resistances and were connected to independent reservoirs that were set at the same hydrostatic level (ie, no flow gradient) [17,19]. The passive luminal diameter was recorded and used as the maximal dilator capacity.

Using the isolated arteriole preparation, experiments were done to examine the vasoactive responses to NE, Ach, and flow-induced mechanical stimulation of the endothelium. After a

Table 1
Body weight and Cu status indices of rats fed the 3 Cu concentrations for 6 months

Parameter	Dietary copper (mg Cu/kg diet)		
	6.0 (n = 9)	3.0 (n = 9)	1.5 (n = 8)
Body weight (g)	650 ± 26	625 ± 27	693 ± 26
Liver Cu (μg/g dry weight)	11.75 ± 0.38	11.66 ± 0.29	11.39 ± 0.58
Kidney Cu (μg/g dry weight)	25.30 ± 1.36	24.29 ± 1.06	20.15 ± 0.85*

Values are mean ± SEM.
  * $P < .05$ compared with 6.0 control diet.

1-hour equilibration period, the baseline luminal diameter was measured followed by constriction to 10^{-5} mol/L NE in the bath. The vessel bath was washed 3 times with MOPS-PSS and allowed to re-equilibrate. Dilation to 10^{-6} mol/L Ach in the bath was then determined and the vessel bath replaced with fresh MOPS-PSS (×3). Flow-induced vasoreactivity was determined by measuring the change in luminal diameter during a pressure gradient between the cannulas ($\Delta P = 40$ cm H_2O). Finally, NO-independent dilation was determined with 10^{-4} mol/L adenosine. The data are presented as a percentage of the maximal diameter for each vessel.

2.4. Statistics

Data are presented as mean ± SEM. Comparisons between dietary groups were by 1-way analysis of variance. The relationship between vasoactive response and kidney Cu concentration was determined by linear regression. Effects were considered different if $P < .05$.

3. Results

Marginal restriction of dietary Cu did not affect the growth of the rats as indicated by comparisons of weight between the dietary groups (Table 1). The diets also did not cause a

Table 2
Comparison of baseline and maximal diameters and vasoreactivity between dietary groups

Parameter	Dietary copper (mg Cu/kg diet)		
	6.0 (n = 9)	3.0 (n = 9)	1.5 (n = 8)
Baseline diameter (μm)	103.3 ± 3.8	100.8 ± 5.8	104.9 ± 5.4
Maximal diameter (μm)	176.6 ± 5.5	175.9 ± 3.5	180.7 ± 3.7
Constriction to NE, 10^{-5} mol/L (% maximal diameter)	22.2 ± 1.7	21.7 ± 2.4	18.9 ± 2.7
Dilation to adenosine, 10^{-4} mol/L (% maximal diameter)	97.0 ± 1.2	95.4 ± 1.7	97.4 ± 1.0
Dilation to Ach, 10^{-6} mol/L (% maximal diameter)	92.4 ± 2.9	94.0 ± 1.4	95.7 ± 1.3
Dilation to increased flow ($\Delta P = 40$ cm H_2O) (% maximal diameter)	68.3 ± 3.5	69.1 ± 2.4	69.4 ± 3.6

Values are mean ± SEM. No value at dietary Cu levels of 3.0 or 1.5 differed from that at 6.0 mg Cu/kg diet.
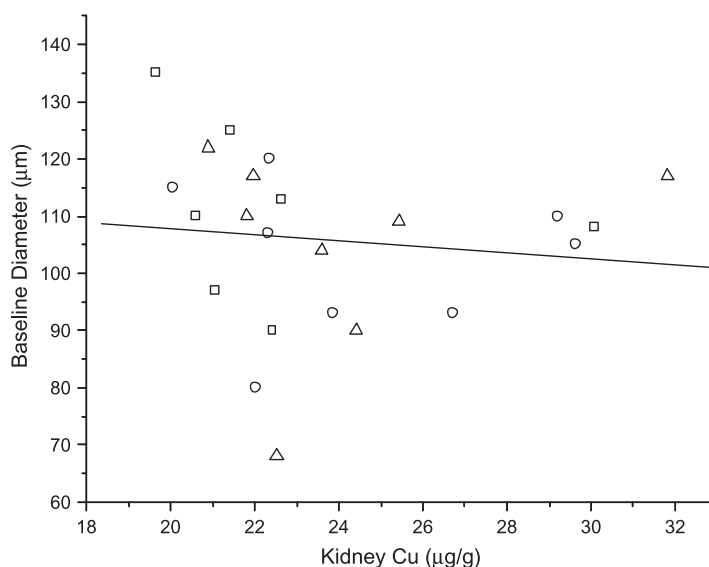
Fig. 1. The relationship between baseline diameters vs kidney Cu concentration for the individual rats. Symbols represent different dietary Cu concentrations (□ = 1.5, △ = 3.0, ○ = 6.0 mg Cu/kg diet). The correlation coefficient is $r = 0.134$ and the significance is $P = .543$.

significant change in liver Cu concentration (Table 1), which is a commonly used index of Cu status. However, the kidney Cu concentration was significantly less in the $CuM_{1.5}$ group compared with the $CuA_{6.0}$ control group (Table 1).
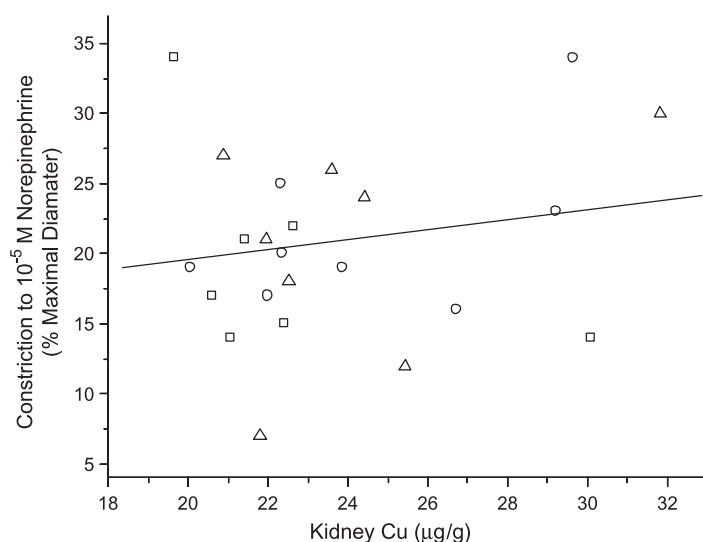

Fig. 2. Constriction of arterioles in response to NE (10^{-5} mol/L) vs kidney Cu concentration for the individual rats. Symbols represent different dietary Cu concentrations (□ = 1.5, △ = 3.0, ○ = 6.0 mg Cu/kg diet). The correlation coefficient is $r = 0.186$ and the significance is $P = .391$.
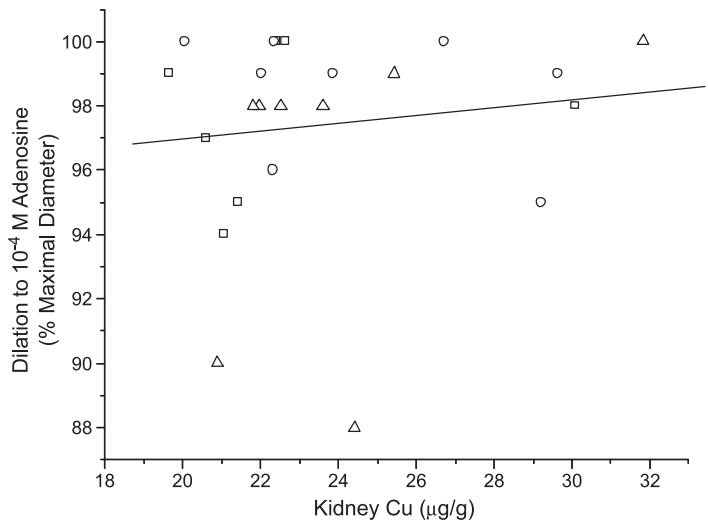
Fig. 3. Dilation of arterioles in response to adenosine (10^{-4} mol/L) vs kidney Cu concentration for the individual rats. Symbols represent different dietary Cu concentrations ($\square$ = 1.5, $\triangle$ = 3.0, $\bigcirc$ = 6.0 mg Cu/kg diet). The correlation coefficient is r = 0.135 and the significance is P = .539.

The different levels of dietary Cu did not affect either the baseline arteriole diameters or the maximal diameter in these resistance vessels. These results are seen in both the comparisons between dietary groups (Table 2) and in the relationship between kidney Cu concentration and baseline luminal diameter (Fig. 1). In the vasoreactivity protocols, the change in luminal diameter caused by each stimulus was compared by dietary group and was
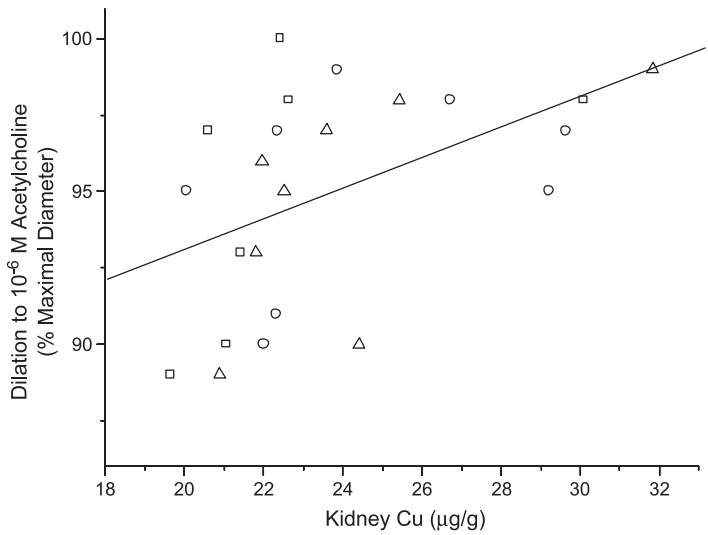

Fig. 4. Dilation of arterioles in response to Ach (10^{-6} mol/L) vs kidney Cu concentration for the individual rats. Symbols represent different dietary Cu concentrations ($\square$ = 1.5, $\triangle$ = 3.0, $\bigcirc$ = 6.0 mg Cu/kg diet). The correlation coefficient is r = 0.484 and the significance is P = .019.
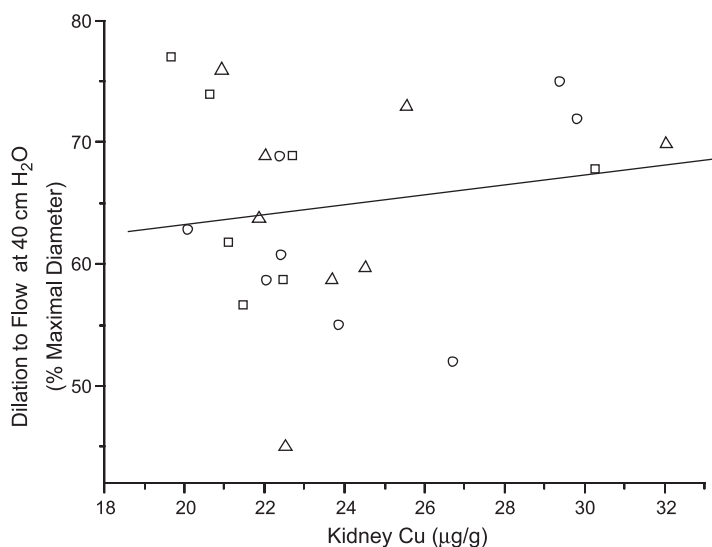
Fig. 5. Dilation of arterioles in response to flow ($\Delta P = 40$ cm H_2O) vs kidney Cu concentration for the individual rats. Symbols represent different dietary Cu concentrations ($\square$ = 1.5, $\triangle$ = 3.0, $\bigcirc$ = 6.0 mg Cu/kg diet). The correlation coefficient is $r = 0.134$ and the significance is $P = .543$.

plotted vs the kidney concentration for each individual rat to give a clearer assessment of the effect of the marginal Cu status. There was no statistical difference in vasoreactivity between any of the dietary group to any of the stimuli used (Table 2). In addition, there was no correlation between NE (10^{-5} mol/L)-induced constriction and kidney concentration of Cu (Fig. 2; $r = 0.186$, $P = .391$) or between dilation to NO-independent adenosine (10^{-4} mol/L) (Fig. 3; $r = 0.135$, $P = .539$). However, the dilation to Ach (10^{-6} mol/L) in each arteriole was significantly correlated to the kidney concentration of Cu for that animal (Fig. 4; $r = 0.484$, $P = .019$). Mechanical stimulation of the vascular endothelium with flow caused dilation of the vessels but the response did not correlate to the kidney Cu concentration (Fig. 5; $r = 0.134$, $P = .543$).

4. Discussion

As anticipated, group mean organ Cu concentrations of adult, marginally Cu-deficient rats were relatively insensitive to dietary Cu concentration, similar to findings for young animals [13]. Only kidney Cu showed a significant response to marginal deficiency and only for rats fed a diet of 1.5 g/kg Cu. It was therefore not surprising that group mean values of vasoactivity measurements, less direct indicators of Cu status than organ Cu, also showed no relationship to dietary Cu concentration. From this it is apparent that, at marginal intakes, Cu status cannot be inferred only from the diet concentrations the animals consume. Greater sensitivity in status assessment requires that inter-individual variation in the way each animal consumes, absorbs, and metabolizes a nutrient must be taken into account. This potential for inter-animal variability was addressed in prior studies [7,13,20,21] by plotting each animal's Cu status (eg, organ Cu) against the variable of interest and testing for significance of

correlation. For the particular case of marginal deficiency in young animals, using kidney Cu in this correlative technique proved to be more sensitive than either liver Cu or activity of serum ceruloplasmin (a Cu-dependent enzyme) in detecting variables affected by marginal Cu status [13]. We suspected that such use of kidney Cu as an index would be useful in the present study.

Measurements of kidney Cu in this study quickly confirmed our previous view [13] that a wide range of kidney Cu concentrations is expressed in a group of animals consuming a given dietary Cu concentration, a range that in fact overlaps those of animals consuming other diets. We believe that this underlines the need to distinguish between animals on the basis of more than just the diets they eat.

Use of regression analysis demonstrated a significant correlation between kidney Cu concentration and Ach-induced dilation but no relationship between kidney Cu and NE-induced constriction, adenosine-induced dilation, or flow-induced dilation. The reactivity changes to Ach were seen in arterioles that had the same basal tone and the same dilator capacity across varying dietary Cu intakes. The results demonstrate that the defect in Ach-induced dilation is specific for the Ach-NO signaling pathway and not a generalized loss of endothelial or vascular smooth muscle function, which is consistent with observations made in vessels of young, severely Cu-deficient animals [5,6] and in Cu-chelated isolated vessels [4,9]. Though not tested here, we anticipate that the mechanism of impaired Ach-NO signaling is the same as that established in more severe deficiencies, specifically, reduction of activity of Cu-dependent superoxide dismutase allows superoxide-mediated oxidative interference with the signaling pathway [3,4,9]. In this scenario, NO effectiveness as a dilator is reduced by its conversion to peroxynitrite and by oxidative impairment of the calcium mobilization required for activation of endothelial NO synthase [8]. The apparent impact of this altered NO-mediated signal transduction in young rats is an impairment of blood pressure regulation [21].

Two key points can be drawn from this study of resistance arterioles. The first is that the Ach-NO transduction pathway appears to be very sensitive to suboptimal dietary Cu. The current study suggests that Ach-induced vasodilation may be compromised although traditional indices of Cu status such as liver Cu are not affected. Although there may be no dietary effect on vascular tone as seen in the baseline diameters, an effect of marginal Cu deficiency on resistance arterioles may be seen as an inability to respond to NO-mediated dilators. Although the effect is subtle, we regard this finding as important because it implicates marginal deficiency at levels consistent with human Cu intake as an effector of vascular function and therefore potentially of blood flow and pressure. The fact that reduced NO availability has been associated with human hypertension [22] makes more compelling the examination of a possible role for impaired Cu status in the etiology of this disease. The observation of an exaggerated rise in blood pressure after exercise stress in otherwise normotensive, marginally Cu-deficient women [23] offers preliminary evidence of this possibility.

Secondly, the results seen in short-term severe Cu deficiency may be predictive of effects of long-term marginal Cu deficiency. The specific inhibition of the Ach-induced dilation without effect on the NE-induced constriction or vasoreactivity in general is the same as is seen in arterioles from studies of weanling, short-term severe Cu deficiency [3]. This finding

gives greater credibility to studies using the short-term severe deficiency model although only marginal Cu deficiency is prevalent in the human population [11,12].

In summary, this study is the first to report a defect in vascular function caused by long-term marginal Cu deficiency in adult animals. This model provides new insight into the role of dietary Cu in vascular function because the dietary Cu restriction was started after the developmental stage of the rat and extended for approximately a fourth of the rat's life span. Although the model was different, the results were the same as previously seen with the weanling short-term rat model of Cu deficiency [7]. The results demonstrate a significant relationship between Cu status and Ach-induced vascular smooth muscle relaxation in resistance arterioles. Furthermore, the results support the use of kidney Cu as a sensitive index of Cu status.

Acknowledgments

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